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Antioxidant, Anticancer and Antibacterial Activity of *Withania somnifera* Aqueous Root Extract

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ROA and PSN jointly designed the study and wrote the protocol. Authors DAB and RB performed all experiments and data analysis. Author DAB wrote the 1st draft. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aims: To evaluate total antioxidant capacity, anticancer activity and antibacterial effects *Withania somnifera* aqueous-root extracts.
Study Design: *In vitro* study.
Place of Study: School of Biomedical Sciences, Ulster University, UK.
Methodology: Total antioxidant capacity (TAC) of whole powder and freeze dried *W. somnifera* aqueous-root extracts was determined using FRAP, DPPH, Folin and ABTS assays. Anticancer activity was accessed using MDA-MB-231 breast cells and Sulforhodamine B staining for cell viability. Antibacterial activity was by disk diffusion assay with penicillin, amoxicillin and streptomycin as positive controls.
Results: The TAC for *W. somnifera* extract was 86, 47, 195,or 443 gallic acid equivalents per 100g dry basis (mgGAE/ 100 g) using FRAP, DPPH, Folin or ABTS assays, respectively. Corresponding TAC values for freeze dried *W. somnifera aqueous-root extract were,* 418, 553, 1898 or, 1770 (mgGAE/100 g). *W. somnifera* aqueous-root extract inhibited MDA-MB-231 cell proliferation in a dose-dependent manner with IC_{50} = 0.19 mg/ml (21 µM GAE). Nil antibacterial effects were detected for freeze dried *W. somnifera* extract (0-1 mg/ml) across six species of bacteria tested.

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Conclusion: *Withania somnifera* root water extract showed significant antioxidant and anticancer activity for MDA-MB-231 breast cancer cells but no antibacterial activity under the conditions of this study.

Keywords: Withania somnifera; antioxidant capacity; anticancer; antibacterial.

1. INTRODUCTION

*Withania somnifera (*Ashwaganda, Indian ginseng or Winter cherry) is a member of the Solanaceae family found naturally in Africa, Asia (temperate/ tropical) and Europe [1]. Health uses of *W. somnifera* can be traced from 5000BCE to Hindu Ayurveda medical practices. Recent chemical analysis showed diverse components, including alkaloids, steroidal lactones, and flavonoids; [2-5]. The in-vitro antioxidant activity of *W. somnifera* roots extracts were determined as part of routine investigations [6-9] with both extracts and isolated components reported to inhibit breast cancer cells [10-14]. The antimicrobial activity of *W. somnifera* extracts received increasing interest over the recent past as shown by representative studies [7,15-19].

Currently, a few studies examined the anticancer effect of root extracts *W. somnifera* [20-22] and comparatively less research dealt with aqueous extracts [23]. To our knowledge, no publication has compared the antioxidant, anticancer and antimicrobial activity for *W. somnifera* within a single study. To fill the perceived research gap, the aims of this study were to evaluate *W. somnifera* aqueous root extracts, for antioxidant, anticancer and antibacterial effects *in-vitro*.

2. MATERIALS AND METHODS

2.1 Materials and Extract Preparation

USDA approved organic *W. somnifera* root power was purchased from a licensed herbal dealer. Chemicals and reagents including 2, 2-diphenyl-1-picrylhydrazyl, Folin Denis reagent and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) were obtained from Sigma-Aldrich. Nutrient broth and nutrient agar were both purchased from Oxoid. Aqueous root extract was prepared by adding 1 g of whole *W. somnifera* root powder to 19 ml of water, and stirring for 1 hour, centrifuging (13000 rpm for 20 minutes), freezing at -80ºC overnight and freeze drying.

2.2 *In vitro* Assay for Total Antioxidant Capacity

Assays for total antioxidant capacity (TAC) were adapted to microplate analysis as described previously [24]. The ferric Reducing Ability of Plasma (FRAP) assay was adapted from [24] and samples were read at 593nm in a microplate reader. The Folin assay for total phenol was performed as described in [25] and read at 760nm using a microplate reader. The DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay was adapted from [26] and samples were read at 515nm using a microplate reader. The ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) assay was adapted from [27] and the final solution mixtures measured at 734nm using a microplate reader. All assays were calibrated with 0-3 mM gallic acid as reference antioxidant.

2.3 Anticancer Assay

MDA-MB-231 breast cancer cells were grown in DMEM with 10% FBS, 1% PenStrep and 1% non-essential amino acids. Trypsinized cells were plated (10,000 per well) and allowed attach overnight. Freeze dried *W. somnifera* (5 mg/ml) in complete culture medium was filter sterilized and 1:5 serial dilutions were added to cells for 72 hrs. Cells viability was determined using sulforhodamine B staining [28].

2.4 Antibacterial Methods

Three Gram positive (*B. cereus, S. aureus, S. epidermis)*, and Gram negative (*Salmonella typhi, E. coli and Pseudomonas aeruginosa) with verified,* identity and phenotypes were used. Antimicrobial activity was evaluated by disk diffusion assay as described by [17]. Control antibiotic disks contained either streptomycin, amoxicillin or, penicillin; two blank discs were placed, at equal distances on the agar plate. Freeze dried plant extract (1 mg/ml) was added to discs one drop at until the disk was saturated.

2.5 Statistical Analysis

Statistical analysis was carried out using Microsoft excel and the IBM SPSS v22 statistic

package for Microsoft Windows for ANOVA analysis. Significance level was at (*P*=0.05) across all tests.

3. RESULTS AND DISCUSSION

3.1 Total antioxidant Capacity Assay

The total antioxidant capacity (TAC) for *W. somnifera root* aqueous-extract and the freeze dried extract (Table 1) reflected the method of analysis (ABTS > Folin > FRAP ≥ DPPH) and values were significantly different for all assays (p=0.05). The total phenols values (95 & 1898 mg GAE/100 g) for whole water extract and freeze dried extract, compare with 60 -1107 mg GAE/100 g reported previously [6,7]. The total phenols value for the *W. somnifera* root extracted with 80% hot methanol was 600 mg GAE/100 g [9]. The FRAP values (86 - 418 mg GAE/100 g) compares well with 167-200 mg GAE/100 g (converted from 500-600 mg trolox equivalent/ 100 g) reported for *W. somnifera* methanolic extract [9]. ABTS analysis for *W. somnifera* root water-extract produced a TAC of 218 mgGAE/100 g (from 938 mg trolox equivalents/ 100 g) [7] compared with 443 and 1770 mg GAE/100 g from this study. In summary, the TAC for *W. somnifera* is influenced by the extraction conditions (solvent used, temperature and time of extraction), plant maturity and botanical part of the plant selected. *W. somnifera* leaves had 3-4 times higher phenols and TAC compared with roots for the fully mature plant [9] whilst other investigators found no systematic difference [8,17]. The present paper provides a unique comparison of TAC for the *W. somnifera* root extracts using four different assays, which is important because different antioxidant methods employ different mechanisms [29].

3.2 Anticancer Activity towards MDA-MB-231 Breast Cancer Cells

Breast cancer is the most common cancer among women globally with 1.67 million new cases diagnosed in 2012 and accounting for 25% of all cancers [30]. *W. somnifera* leaf extracts and isolated components were reported to inhibit breast cancer cells [10-14] but few studies examined the anticancer effect of root extracts [20-22] and studies using aqueous-extracts are limited [23]. In the present study (Fig. 1), freeze

Table 1. Antioxidant activity for whole powder (WP) and freeze dried *Withania somnifera* aqueous extracts across four *in-vitro* antioxidant assays

Assay*	LDR (µM)	ε (M cm^{-1})	R^2	TAC ‡(WP)	TAC‡(FD)
FRAP	0-25	195014	0.997	86±11	418 ±12
DPPH	0-25	92886	0.989	47±8	553.1±31
Folin Denis	0-75	25836	0.981	195±11	1898±57
ABTS	0-20	50642	0.988	443±28	1770±57

Results from 3-replicates, n ≥48 data points, WP = whole powder, FD = freeze dried extract
**LDR =Range of concentrations or which calibration graph is straight, ε (M cm^{-1}) = molar absorptivity, R^2 = Correlation coefficient. ‡Total antioxidant capacity (TAC) expressed as gallic acid equivalents (GAE) per 100g dry weight. Within each column all TAC are significantly different at (P=0.05)*

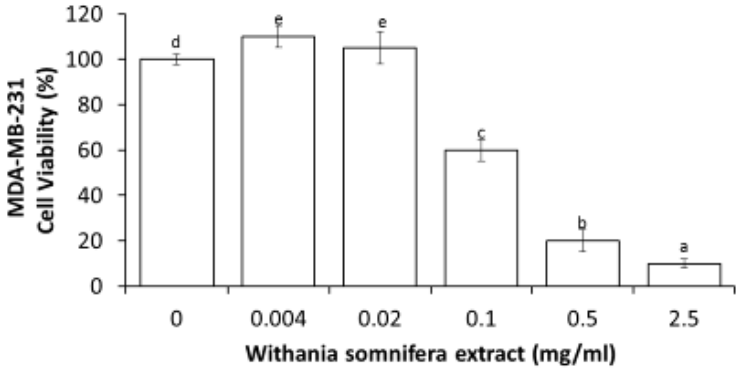

Fig. 1. Effect of different doses of aqueous extracts from *W. somnifera* root powder (0.004, 0.02, 0.1, 0.5, 2.5 mg/ml) on MDA-MB-231 cell viability with 72hr treatment
Bars shows means (+/- SEM) for 3 independent experiments (n= 18) per treatment, Different letters on each column show significant differences (P=0.05)

dried *W. somnifera aqueous* root extracts were found to be cytotoxic to MDA MB 231 breast cancer cells with 50% inhibitory concentration (IC50) at 0.19 mg/ml for 72 hr. treatment. Cell viability was just over 10% at the highest concentration used. By comparison, a previous report found the IC50 was 0.48 mg/ml and 0.032 mg/ml for aqueous and ethanoic extracts from the roots of *W. somnifera* tested with MDA-MB-231 cell line though there were detailed differences in experimental method [23]. There is general agreement that both leaf and root extracts from *W. somnifera* possess anticancer activity [31]. Interestingly a previous study showed that leaf and root extract had similar anticancer activity though more exhaustive studies are needed [14].

Weather the anticancer and TAC activity of *W. somnifera* are related is uncertain To address this issue, we estimated the IC50 value for freeze dried *W. somenifera extract* in terms of total phenols content of samples (IC50 (mol/l) = 0.19 (gGAE/l) * 18.89 (gGAE/g sample)/ 170.1 (g/mol)) which is 21 µM GAE. Interestingly, the IC50 for pure gallic acid for MDA-MB-231 cells was 47 µM with 24 hr treatment [32].

3.3 Antibacterial Activities

Previous investigations found, that *W. somnifera* extracts show antimicrobial activity towards both Gram negative and Gram positive strains but, that the degree of effectiveness varied with plant components (leaf > root extracts), extraction solvent (methanol, chloroform > water > petroleum ether) and dose of bioactive agent applied [7,15-19]. The present study (Fig. 2) show that *W. somnifera* aqueous-root extract had no antimicrobial effects at the dose tested (1mg/ml; 1mg addition/disc). Of particular note, previous investigations using disc diffusion testing required a concentration range of 20-100 mg/ml of extract with 4-5 mg solid per disc or well [16,17]. A methanolic *W. somnifera* root extract (5mg/ml and 200 µl or 1 mg addition per disc) showed antimicrobial activity but lower concentrations were not tested [8]. Aqueous and chloroform extracts of *W. somnifera* fruit extracts (10 mg/ml; 100 µl or 1 mg addition per disc) showed antimicrobial results [33]. Finally, recent investigations using 100 mg/ml extract (10 mg addition/ disc) found aqueous, ethanol and acetone extracts of *W. somnifera* had broadly similar antimicrobial activity [17,34].

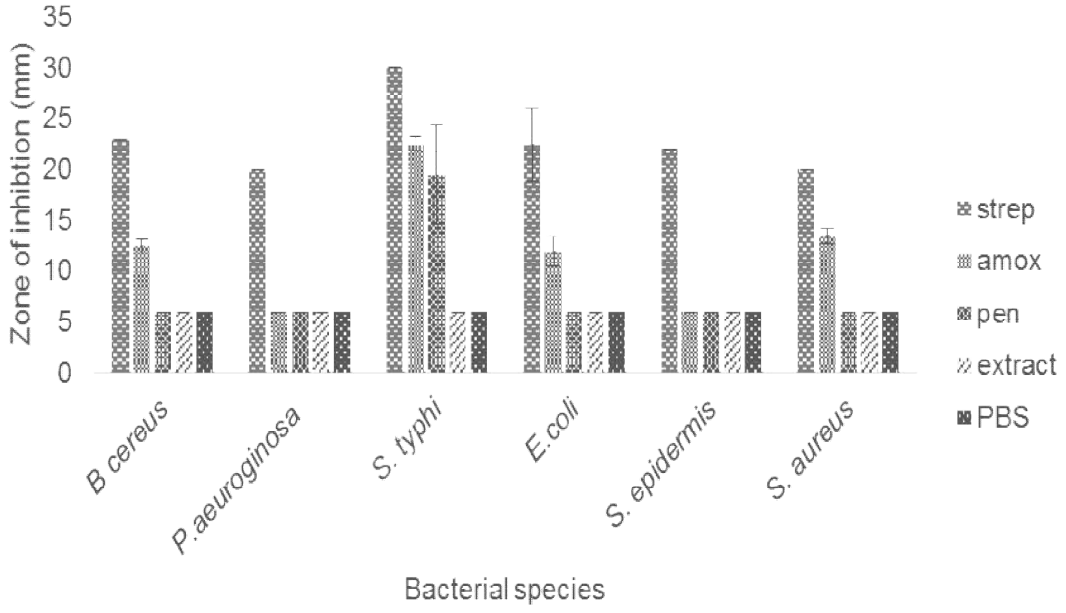

Fig. 2. Zones of inhibition for disc diffusion assay of *Withania somnifera* extract using Gram +ve (*B. cereus, S. aureus S. epidermis*) and Gram -ve *(Salmonella typhi, E. coli and Pseudomonas aeruginosa 17)* bacteria

Extract 1 mg/ml (1 mg/ disk), 3 control antibiotics and PBS as an additional control. Error bars are standard deviation. 6 mm is size of antibiotic disk. Number of replicates n=2

4. CONCLUSION

Withania somnifera aqueous root extract was demonstrated to have potent antioxidant activity. Freeze drying the *W. somnifera* root extract further increased the TAC per dry weight basis by 5-11 fold and is expected to also preserve antioxidant activity. Aqueous-root extracts *W. somnifera* showed anticancer activity (IC50~0.19 mg/ml) but no antibacterial ability was detectable at such doses. This preliminary study is the first to compare, antioxidant, anticancer and antimicrobial activity of *W. somnifera* extracts. The results, supported by emerging literature, suggest that the effective dose for *W. somnifera* anticancer activity is 1-order of magnitude lower compared to concentrations for antimicrobial activity according to the strain of bacterial tested in this study. Further research is required to improve current understanding of the anticancer and antimicrobial properties Withania.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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